

Anti-inflammatory effect of *Leucas cephalotes* Spreng in 12-O-tetradecanoylphorbol-13-acetate-induced inflammation in mice

Kuldeep Singh Yadav*, Narayan P. Yadav*, Naveen Kumar* and Suaib Luqman**

*Herbal Medicinal Product Department, CSIR- Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, U.P., India

**Molecular Bioprospection Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, U.P., India

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Abstract

Leucas cephalotes Spreng (Family: Lamiaceae) known as 'Dronapushpi' in Sanskrit and 'Guma' in Hindi, is being extensively used by rural people of North India to treat various ailments including inflammation. Therefore, present study was undertaken to validate the effect of *L. cephalotes* extracts in topical inflammation, using mouse ear edema model, induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). The anti-inflammatory effect was evaluated by measuring ear weight, level of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , interleukin (IL)-6, activity of lipid peroxidation (LPO) and the production of nitric oxide (NO). The results showed that non-polar fraction of methanolic extract (MNF) of *L. cephalotes* significantly decreased the ear weight, LPO activity and NO level. Additionally, MNF remarkably inhibited the expression of TNF- α , IL-1 β and IL-6 while polar fraction of methanolic extract (MPF) considerably reduced LPO activity and IL-6 level only. The results indicate that non-polar fraction of methanolic extract (MNF) of *L. cephalotes* has potential anti-inflammatory effect on TPA-induced inflammation in mice ear.

Key words: *Leucas cephalotes* Spreng, TPA, Anti-inflammatory activity, Proinflammatory mediators, Lipid peroxidation, Nitric oxide

Introduction

Inflammation is a part of complex biological response of vascular tissues to harmful stimuli like pathogens, damaged cells, traumas, bone fracture, hypersensitivity reactions or irritants. Aberrant activation of the acute inflammatory response can damage various tissues and organs which lead to chronic inflammation. Persistent chronic inflammation may cause life threatening diseases such as bacterial sepsis, rheumatoid arthritis, skin inflammation and cancer (Palladino *et al.*, 2003 and Paul *et al.*, 2012). During recent years, there has been a resurgence of interest in the study of mechanisms of inflammation related to cancer and in the use of such mechanisms as the basis for development of new

chemopreventive agents. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Now-a-days, toxicity and revival of symptoms are common problems for anti-inflammatory drugs which are primarily of synthetic origin. These untoward effects restrict their long-term use. Hence, there is a constant demand for better therapeutic alternatives. Some of the plants with anti-inflammatory activity have been scientifically analyzed (Delporte *et al.*, 2005) because the medicines based on plant origin are promising choice over modern synthetic drugs. They show minimum/no side effects and are considered to be safe (Miyachi *et al.*, 2006). In search of new anti-inflammatory lead molecule, it is better to screen plants traditionally used for inflammatory conditions.

Leucas cephalotes Spreng. is an annual herb found on waste lands throughout India (Dua *et al.*, 2011). Phytochemical investigations show the presence of flavonoids, phenol, phytosterol and tannins (Baburao *et al.*, 2010). The decoction of dried aerial parts of plant is used orally for diarrhoea (Pushpangadan and Atal, 1984), to reduce fever (Johns *et al.*,

Author for correspondence: Dr. Narayan Prasad Yadav
Herbal Medicinal Product Department, CSIR- Central Institute of
Medicinal and Aromatic Plants, Lucknow-226015, U.P., India

E-mail: npyadav@gmail.com

Tel.: +91-522-2718657, **Fax.:** +91-522-2342666

+91-09451244418, +91-09450904995

1995), as an appetizer (Johns *et al.*, 1990), an emmenagogue and for coughs and colds (Pushpangadan and Atal, 1984). The flowers and leaves are applied externally as poultice to treat headache (Saha and Kasinathan, 1961). The decoction of flower heads in Nepal is used orally to treat jaundice (Bhattarai, 1997). The juice of unripe fruits is used externally to treat scabies (Holdsworth, 1991). The juice of leaves is used externally as well as nasally as an antivenom (Reddy *et al.*, 1989). The dried leaves are used orally as a blood purifier (Das *et al.*, 2012). Beside these, *L. cephalotes* has been reported for antioxidant, analgesic and anti-inflammatory (Baburao *et al.*, 2010), antidiabetic (Bavarva and Narasimhacharya, 2010), antiprotozoal (Dua *et al.*, 2011) activities. Recently, Baburao *et al.* (2010) have reported the anti-inflammatory effect of methanolic extract from *L. cephalotes* on paw edema volume in carrageenan-induced rat model only at dose 200mg/kg and 400mg/kg body weight but their investigation does not reveal the mechanism behind reducing inflammation. Therefore, the present study was designed to explore and validate the topical anti-inflammatory effect of *L. cephalotes* using TPA in mouse ear edema and revealing the mode of action of the same by determining the expression proinflammatory cytokines.

Materials and Methods

Chemicals and reagents

12-*O*-tetradecanoylphorbol-13-acetate (TPA), Indomethacin, Thiobarbituric acid (TBA), Malondialdehyde (MDA), Trichloroacetic acid (TCA), Griess reagent kit were procured from Sigma-Aldrich (USA), biochemical kits for TNF- α , IL-6 and IL-1 β from Thermo Scientific (USA) and Phosphate buffer saline (PBS) tablets were purchased from Himedia, Mumbai. All other chemicals and reagents used in the study were of analytical grade.

Plant material

Fresh plant of *Leucas cephalotes* Spreng was collected from nearby area of Lucknow (Uttar Pradesh), India. The material was authenticated by taxonomist, Dr. S.C. Singh and a herbarium was deposited in Botany Department of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow (Voucher Specimen No. 9578). Plant material (1kg) was dried in shade, powdered using mixer grinder and stored in an airtight container.

Preparation of extracts and fractions

Dried powder (100g) was extracted with methanol and water separately by maceration for approximately 72h with occasional shaking. After filtration both the extracts were concentrated using rotary evaporator under reduced pressure, dried at 40°C on water bath and yields were recorded for methanolic (5.34%) and aqueous extracts (AQE; 5.04%). Methanolic extract was further fractionated with petroleum

ether to separate polar fraction (MPF; yield 53.15%) and non-polar fraction (MNF; yield 46.85%).

Study design

Before inducing inflammation, mice were grouped into six distinct groups having 6 animals in each group (n=6). Ear edema was induced in the right ear of each mouse by topical application of TPA as previously described (Xian *et al.*, 2011b) with a slight modification. Briefly, 20 μ l of TPA solution (1 μ g/20 μ l of TPA in acetone) or vehicle (acetone) was topically applied to the both inner and outer surfaces of the right ear after every 24h for 5 days. Extracts of *Leucas cephalotes* viz., AQE, MNF and MPF each in concentration of 10mg/20 μ l in acetone and indomethacin (0.5mg/20 μ l in acetone, used as positive control) were applied topically 30 min after TPA treatment for 5 days. Redness of right ear of each animal was observed daily and scored arbitrary from 0 to 5 (1-Very low, 2-Low, 3-Mild, 4-High, 5-Very high; Chang *et al.*, 1987). After 2h of the last treatment with TPA, mice were sacrificed and each ear (right and left) biopsies were obtained with a punch (8 mm diameter) and weighed immediately. The edema was measured in terms of weight difference of the ears (Rasadah *et al.*, 2004). Further, 50 mg ear tissue was minced and homogenized in 1ml of phosphate-buffered saline (pH 7.4) using tissue homogenizer. The homogenate was centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was collected in separate micro centrifuge tube and stored at -80°C until biochemical tests were carried out.

Cytokines estimation

For determination of cytokines, methodology of Xian *et al.* (2012) was used with slight modifications. In brief, the part biopsies of 8mm ear punch were homogenized vigorously in 1ml ice cold PBS buffer (pH 7.4). After incubation in ice for 15min, the homogenate was centrifuged at 10,000 x g for 20 min at 4°C. The levels of TNF- α , IL-1 β and IL-6 in the supernatants were measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (Thermo Scientific, USA) according to the manufacturer's instructions.

Lipid peroxidation (LPO) assay

Lipid peroxidation was estimated according to Shafiq-ur-Rehman *et al.* (1995) by measuring malondialdehyde (MDA) levels based on the reaction of MDA with Thiobarbituric acid (TBA). Briefly, to 200 μ l of supernatant, equal volumes of 10% w/v TCA was added and mix well to precipitate the protein content. The mixture was centrifuged at 5,000 rpm for 5 min and aliquot of 200 μ l supernatant was reacted with equal volume of 200 μ l of 0.67% of thiobarbituric acid (TBA). The above sample was kept in boiling water bath for 10 min. Blank was prepared only with TCA and TBA reagent. The absorbance was measured at 535nm and 600nm and estimation of LPO was done using standard plot.

Estimation of nitric oxide concentration

The level of NO was estimated by measuring nitrite concentration in tissue homogenate as described by Xian, Li *et al.* (2011a). About 75µL of ear tissue supernatant was taken in a micro titer plate and mixed with equal volume of griess reagent and incubated at 37°C for 10 min. The absorbance was read out at 540nm using a spectrophotometer. The concentration of nitrite was calculated from a standard curve plotted with serial dilutions of sodium nitrite. Concentration of nitrite in samples was calculated using following formula:

Nitrite concentration (µM/ml)

$$= \frac{\text{Absorbance of test} \times \text{Concentration of standard}}{\text{Absorbance of standard}}$$

Statistical analysis

All the values expressed are Mean ± Standard error (SE). The data was statistically analyzed by one-way-ANOVA following Tukey's Post-hoc test using GraphPad PRISM® version 5.01 (GraphPad Software, Inc., USA). The differences were considered significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results and Discussion

The present study is the first report to determine effect of *Leucas cephalotes* extracts in TPA-induced inflammation in mouse model. TPA-induced mouse ear edema is an excellent animal model for inflammation which is closely related with the infiltration of macrophages and neutrophils, induction of proinflammatory cytokines including TNF-α, IL-6, IL-1β and generation of nitric oxide. Therefore, it can be very useful short-term test to detect the agents with anti-inflammatory potential (Murakami *et al.*, 2000). Topical application of TPA increased ear redness by 3.4 fold and edema (ear weight) by 16.5 fold as compared to vehicle control group. Redness and edema were reduced significantly *i.e.*, 50% ($p < 0.01$) and 54.1% ($p < 0.05$), respectively by MNF and 41.7% ($p < 0.01$) and 45.8% ($p < 0.05$), respectively by MPF whereas AQE suppressed redness and edema 16.7% and 33%, respectively as compared to toxin control group as shown in Table 1. The results clearly indicate that MNF and MPF are effective in reducing edema.

It is well known that 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes mouse skin carcinogenesis, which is closely linked to inflammatory responses. The inflammatory process involves the activation of monocytes and macrophages, which secrete inflammatory mediators including nitric oxide (Lee *et al.*, 2009). This leads to oxidative stress which also damages other tissues and organs (Halliwell and Gutteridge 1990). Since NO level is important in the assessment of the extent of inflammation and cell viability therefore, the inhibitory effect of *Leucas cephalotes* extracts on NO production level was investigated. Tissue NO level was

increased approximately 4 fold (Table 2) in toxin control mice as compared to vehicle control but topical application of MNF significantly reduced it by 44% ($p < 0.001$) while MPF and AQE reduced only 24% and 5%, respectively as compared to toxin control group. These results suggest that MNF is capable to inhibit the excessive production of NO which could be considered as a promising agent for therapeutic intervention of inflammatory condition.

To study the protective effects of *Leucas cephalotes* extracts on tissue oxidative damages in inflamed mice ear, MDA level as a marker of lipid peroxidation was also measured in ear tissue homogenates. MDA level was elevated in toxin treated mice by 7.1 fold as compared to vehicle control, but significant reduction was calculated in all test groups *i.e.*, MNF, MPF and AQE as 72.0 ($p < 0.001$), 25.9 ($p < 0.01$) and 17.9% ($p < 0.05$) respectively as compared to toxin control group (Table 2).

Owing to inflammatory responses, the activation of the complement cascade results in the recruitment of neutrophils and macrophages, which secrete chemotoxic materials and proinflammatory cytokines such as TNF-α, IL-1β and IL-6 (Cho *et al.*, 2007) which play important role in the regulation of inflammation (Xian *et al.*, 2012). TNF-α drives acute inflammation and inflammatory cell infiltration in tissues (McInnes and Schett, 2007) and IL-1β is a potent mediator in response to infection and injury (Li *et al.*, 2008) while IL-6 plays dual role as proinflammatory and anti-inflammatory. Therefore, inhibiting the over production of these inflammatory mediators may have beneficial effect on these inflammatory diseases (Lee *et al.*, 2012). The increased TNF-α (4.7 fold) and IL-1β (4.8 fold) levels in the tissue homogenate as compared to vehicle control were not significantly influenced by MPF (34.6 and 21.8%, respectively) and AQE (35.1 and 23.5%, respectively) treated mice, while topically applied MNF significantly reduced the increased level of the same cytokines by 43.2% ($p < 0.05$) and 32.2% ($p < 0.05$), respectively (Table 3). Expression of IL-6 was significantly reduced by MNF (24.4%; $p < 0.01$) and MPF (19.3%; $p < 0.05$) while AQE (1.3%) did not influence it. These results indicate that MNF significantly ameliorate chronic inflammation by blocking the increased levels of TNF-α, IL-1β and IL-6.

Conclusion

In the present investigation, it is concluded that the non-polar fraction of methanolic extract (MNF) of *Leucas cephalotes* exhibited significant anti-inflammatory effect in the mouse ear edema induced by TPA. The anti-inflammatory effect of MNF occurs by reducing the ear edema and down-

regulating the expression of LPO, NO, TNF- α , IL-1 β , and IL-6. These results indicate that MNF could be potentially used for the development of a topical agent for the management of inflammatory conditions.

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Table 1: Observational parameters: Degree of redness in the right ears of mice and difference in the weight (mg) of right and left ear punch

Groups	Degree of redness	Difference in the weight
Vehicle control	1.4 \pm 0.24	3.96 \pm 1.84
Toxin control (TPA)	4.8 \pm 0.21 ^{###}	66.22 \pm 0.81 ^{###}
Standard Control (Indomethacin)	2.2 \pm 0.24 ^{***}	29.14 \pm 1.63 [*]
MNF	2.4 \pm 0.52 ^{**}	30.42 \pm 1.20 [*]
MPF	2.8 \pm 0.54 ^{**}	35.88 \pm 0.62 [*]
AQE	4.0 \pm 0.31	44.36 \pm 2.40

Data is expressed as Mean \pm SE, n=6.

^{###}p < 0.001 compared to vehicle control mice, *p < 0.05, **p < 0.01, ***p < 0.001 compared to toxin-treated mice. Where MNF = Non-polar fraction of methanol extract, MPF = polar fraction of methanol extract, AQE = Aqueous extract

Table 2: Oxidative parameters: Level of lipid peroxidation (LPO) and Nitric Oxide (NO) (μ M/ml) in mouse ear tissue homogenate

Groups	LPO Level	NO Level
Vehicle control	16.3 \pm 1.30	3.6 \pm 0.31
Toxin control (TPA)	115.3 \pm 5.41 ^{###}	14.3 \pm 0.43 ^{###}
Standard control (Indomethacin)	29.0 \pm 3.83 ^{***}	5.58 \pm 0.51 ^{***}
MNF	32.3 \pm 3.32 ^{***}	8.07 \pm 0.55 ^{***}
MPF	85.4 \pm 7.41 ^{**}	10.8 \pm 1.80
AQE	94.7 \pm 3.54 [*]	13.6 \pm 0.92

Data is expressed as Mean \pm SE, n=6.

^{###}p < 0.001 compared to vehicle control mice, *p < 0.05, **p < 0.01, ***p < 0.001 compared to toxin-treated mice. Where MNF = Non-polar fraction of methanol extract, MPF = polar fraction of methanol extract, AQE = Aqueous extract

Table 3: Cytokines level: TNF- α , IL-1 β and IL-6 (pg/ml) in mouse ear tissue homogenate

Groups	TNF- α	IL-1 β	IL-6
Vehicle control	25.8 \pm 5.11	23.4 \pm 2.43	19.2 \pm 2.62
Toxin control (TPA)	122.5 \pm 4.53 ^{###}	111.7 \pm 5.41 ^{###}	109.2 \pm 2.54 ^{###}
Standard control (Indomethacin)	55.7 \pm 9.30 ^{**}	38.1 \pm 9.50 ^{***}	27.1 \pm 2.21 ^{***}
MNF	69.6 \pm 15.52 [*]	75.7 \pm 4.80 [*]	82.6 \pm 5.94 ^{**}
MPF	80.1 \pm 3.12	87.3 \pm 5.31	88.13 \pm 4.41 [*]
AQE	79.5 \pm 5.32	85.4 \pm 1.24	107.8 \pm 3.60

Data is expressed as Mean \pm SE, n=6.

^{###}p < 0.001 compared to vehicle control mice, *p < 0.05, **p < 0.01, ***p < 0.001 compared to toxin-treated mice. Where MNF = Non-polar fraction of methanol extract, MPF = polar fraction of methanol extract, AQE = Aqueous extract

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